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This project is focused on an animal model of the human disease, systemic sclerosis (SSc), called Tsk2/+. The SSc-like traits in Tsk2/+ heterozygotes are highly penetrant. In addition to a readily apparent skin fibrosis resulting from ECM anomalies, Tsk2/+ mice show autoimmune and inflammatory features that closely resemble human SSc features, making it useful as a pre-clinical model. In this report, we show a clear time dependence on the gene expression in the skin of the Tsk2/+ mice. We have pinpointed at least one candidate gene in the interval for Tsk2/+ and have confirmed the sequence difference between Tsk2/+ and the parent strain, 101/H5. We present preliminary results on the expression of TGFβ mRNA from cells cultured on ECM from Tsk2/+ and WT littermates that suggest a mechanism for the up-regulation of TGFβ seen in the mutant strain. We show that elastin content in the skin, known to be controlled by TGFβ6 and possibly up-regulated in SSc7, is the earliest indicator of tight-skin in the tissue

**15. SUBJECT TERMS**

Animal model, systemic sclerosis, scleroderma, Tsk2/+, fibrosis, gene, genetics, TGFβ

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Table of Contents

INTRODUCTION ..... 4

BODY ..... 4

Milestone 1 ..... 4
Milestone 2 ..... 4
Milestone 3 ..... 5
Milestone 4 ..... 5
Milestone 5 ..... 5
Milestone 6 ..... 6
Preliminary results and research accomplishments ..... 6
1. Sequence of Tsk2/+ region ..... 6
2. Expression of elastin ..... 8
3. Cellular infiltrates in Tsk2/+ mice ..... 9
4. RNA gene profiling ..... 9
5. Functional analysis of Tsk2/+ candidate gene ..... 10

KEY RESEARCH ACCOMPLISHMENTS ..... 12

REPORTABLE OUTCOMES ..... 12

Manuscripts, abstracts, presentations ..... 12
Degrees obtained that are supported by this award ..... 13
Development of cell lines, tissue or serum repositories ..... 13

CONCLUSION ..... 13

REFERENCES ..... 13

APPENDIX ..... 13

**INTRODUCTION**

Tsk2 mice were discovered more than two decades ago when progeny of a 101/H male in an ENU mutagenesis experiment were noted with very tight skin. *Tsk2* is homozygous lethal, similar to the *Tsk1* mouse model of SSc which results from duplication of the fibrillin gene. Tsk1 has been one of the most commonly used models for SSc and therefore has been extensively characterized. The SSc-like traits in Tsk2/+ heterozygotes are highly penetrant. In addition to a readily apparent skin fibrosis resulting from ECM anomalies, Tsk2/+ mice show more autoimmune and inflammatory features than Tsk1/+, and their longer lifespan and immune features that closely resemble human SSc features are ideal for use as a pre-clinical model. The Tsk2 mutation has been bred onto a homogeneous inbred (C57Bl/6, or B6) background in Dr. Blankenhorn's laboratory (our partnering PI on this project). B6.Tsk2/+ mice have many features of the human disease, including tight skin, dysregulated extracellular matrix deposition, and significant autoimmunity. We have found that Tsk2-mediated autoimmune and fibrotic signs develop *progressively* with age and manifest differently in females than males, a phenomenon also observed in human SSc. These SSc phenotypes in B6.Tsk2 mice are all likely due to a single genetic mutation, which remains unidentified. We had proposed to identify the *Tsk2* gene and understand its mechanism of action as outlined in our statement of work (SOW). This mouse affords a unique opportunity to examine the pathways leading to the multiple clinical parameters of fibrotic disease from birth onward.

**BODY**

Milestones were assigned to this proposal, with tasks to be accomplished by each investigator. The **summary** of our progress relative to these tasks is given below, followed by a complete discussion of our work this year.

***Milestone 1*** Identify Tsk2/+ gene:

Task 1 was for the Blankenhorn laboratory to collect DNA for sequencing (Months 1-6), which we have done. We were unable to collect homozygous DNA from Tsk2/Tsk2 homozygous embryos, however, as all the embryos we collected at the eight day stage, the earliest we could do with the help of a microscope, turned out to be either heterozygous Tsk2/+ or +/+ (wild-type, or WT). This collection was attempted from five Tsk2/+ females pregnant by Tsk2/+ males, so that is Tsk2/Tsk2 embryos were viable, we should have collected 25%. We believe that the age of death on the B6 background must therefore be prior to day 8 post-conception. Nevertheless, we proceeded to collect the Tsk2/+ and 101/H (parental strain) DNAs for sequencing, as the 454 sequence analysis can accurately report sequences from both chromosomes in heterozygous samples.

Task 2 (Months 6-12) was to select anchor sequences for Nimblegen chip design, so that chromosome 1 DNA in the Tsk2/+ interval could be sequenced. This was done by our subcontractor at ASRI, Dr. Fen Hu.

Task 3 (Month 6-12): Dr. Hu and her colleagues have hybridized the mouse genomic DNA to the chips and collected Tsk2/+ interval DNA, meeting this target. They have sequenced both Tsk2/+ and 101/H interval DNA.

Task 4: Dr. Hu and her colleagues are assembling sequence data now, and will align the sequences to compare and report all observed polymorphisms. This task is in progress. This task was originally scheduled for year 1, but it will take some portion of year 2 to present the final alignment.

***Milestone 2*** Determination of mechanism of action of Tsk2/+ gene:

Task 1 (Months 18-32): At Drexel, we were to breed Tsk2/+ mice to a knockout mouse with a deficiency in the newly-identified *Tsk2* gene, to determine if either Tsk2 or wild-type allele can complement the genetic deficiency. The Blankenhorn laboratory has purchased and bred three Col3A1 KO male mice to Tsk2/+ dams in July 2012 (month 12). We are expecting pups in 2-3 weeks, so results are not yet available.

Task 2 (Months 1-36). Correlate the known actions of the Tsk2 gene at Drexel with gene expression data at Dartmouth (Aim 2) and with the presence of proliferating cells (Aim 3). In this Task, largely accomplished at Dartmouth with the microarray studies (months 4-12), we will establish the timeline for the gene signatures in male and female Tsk2/+ mice. We will then examine the corresponding Tsk2/KO mice for these phenotypes to detect alterations in the TGFβ 1-driven proliferative, fibrotic signature of the Tsk2/+ gene when it is absent. We expect that TGFβ1 is a necessary component in the disease pathway, so at Drexel, we will breed Tsk2/+ mice to TGFβR conditional KO mice when we fully understand the timeline of the TGFβ signature.

***Milestone 3*** Determine the timing of TGFβ activation in the Tsk2/+ mice, and differences between males and females. Dr. Blankenhorn will send mouse tissues to Dr. Whitfield, who will do the RNA work.

Task 1 (Months 1-36): The Blankenhorn laboratory will breed sufficient numbers of mice to collect skin at postnatal Day 0, day 7, day 14, day 21 as well as 1 month and 4 months. These mice are used by all three investigators, and whenever possible, each individual mouse was studied for the relevant traits in each laboratory, so that histology and RNA transcript analysis will occur on the same animal. We have met our targets in year 1.

Task 2 (Months 4-12) Prepare RNA from skin at Drexel and hybridize DNA microarrays at Dartmouth. Data will be analyzed, processed and stored. In practice, we found it better to send whole skin samples to Dartmouth and prepare the RNA there.

Task 3 (Months 12-36): At Dartmouth perform data analysis for expression of TGFβ as well as other gene signatures, both profibrotic (IL13 and IL4) and those that may not be expected (genome-wide).

Task 4 (12-24 months, if necessary): If the microarray study is unclear, we had proposed a small number of RNASeq runs to validate the gene expression data.

Task 5 (dependent timing): Immunohistochemistry will be performed for the validation of TGFβ signatures found in the microarrays. This will be performed by the Artlett and Blankenhorn laboratories. This study relies on the completion of Aim 2 (Milestones 2 and 3), for which we need microarray data from all ages.

***Milestone 4*** Characterize how well the Tsk2/+ mouse approximates human SSc at different time points.

Task 1: At Dartmouth, map mouse genes to human orthologs, integrate mouse and human data using Distance Weighted Discrimination to remove systematic biases, and cluster mouse and human data (months 12-36).

Task 2: At Dartmouth, Analyze data-driven groupings, pathways, computational validation and data interpretation (months 12-36). Data analysis for expression of proliferative signatures will give us a way to understand the subset of SSc patients that exhibit diffuse clinical symptoms with signs of cell proliferation. This is a special investigation of proliferative signatures by the Whitfield group to capitalize on their extensive experience with cell cycle and proliferative motifs in gene expression. It was scheduled for months 8-24, and is in process.

***Milestone 5*** Experimental (functional) validation.

Task 1: We will perform confirmation qRT-PCR on select genes based on Aim 2 in the Blankenhorn and Whitfield laboratories. We had scheduled this for months 4-24; and thus, we have not completed this task as microarray data from all ages is not yet finished. In retrospect, we will modify this task to extend to year three as well, to ensure full study of interesting gene expression patterns over mouse developmental ages. In the Drexel laboratories, we plan experimentation on the mechanotension of the ECM when it contains Tsk2/+ collagen in comparison to ECM containing WT collagen, after the identification of the Tsk2/+ candidate gene by Aim 1.

***Milestone 6*** Cross-breed Tsk2/+ mice to Wsh mast cell knockout mice (at Drexel).

Task 1 (no date): B6.Tsk2/+ mice will be bred to B6.Wsh (c-kit deficient) mice (Jackson stock # 005051) to further determine the role of mast cells in the TGFβ1 signature observed in the Tsk2/+ mouse. We have reason to think that this will not be necessary, as we have found no difference in the mast cell number between Tsk2/+ mice and their age and sex-matched littermates.

***Preliminary results and research accomplishments***

1. *Sequence of Tsk2/+ region*

We have sequenced the relevant 2 MB interval containing the Tsk2/+ gene (Drexel and ASRI). Tsk2 locus-specific capture from mouse chromosome 1 (43.9-45.9 Mb) from both tight skin-affected an unaffected animals was done using whole genome DNA and a custom Nimblegen capture array designed to provide coverage for all nonredundant sequences within the SSc locus. This array was composed of overlapping long (~70 nucleotides) oligomers that collectively cover the genomic locus (minus redundancies). This custom array provided coverage of the region between nucleotide bases 44,241,285 and 47,116,891 of the mm10_dna build of mouse chromosome 1. Thus, the total locus size is 2,875,605 bp. The capture array has probes that directly cover 56.3% of this region or 1,618,504 bp – representing the nonredundant aspects of the locus. However, the amount of DNA sequence (i.e. total length of the actually captured DNA) from each animals genomic DNA was significantly higher as the DNA fragments being captured are much longer than the probes. This resulted in the production of > 2 Mb of targeted unique sequence for each animal which was generated using the CGS's 454 LifeSciences Titanium platform. The DNA yield and the fold enrichment (based on qPCR) for the four samples prior to sequencing are as follows:

Mouse 2044 = 15.5 ug and 50 fold enrichment

Mouse 2045 = 6.4 ug and 14 fold enrichment

101B = 22.5 ug and 29 fold enrichment

101E = 23.0 ug and 34 fold enrichment

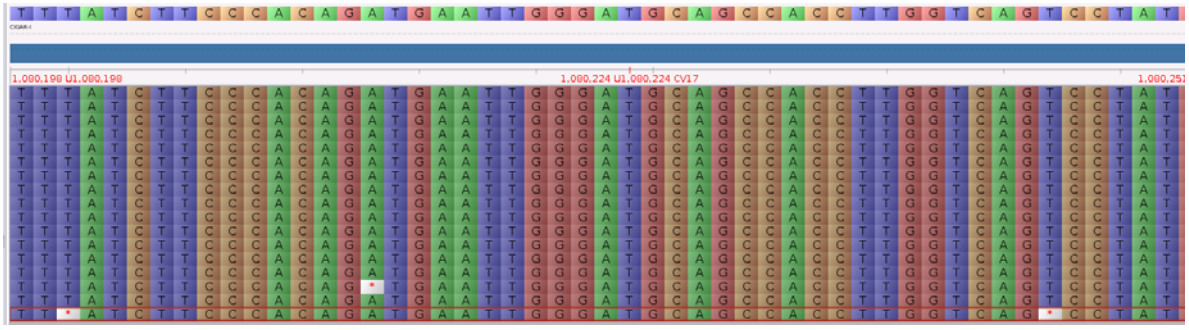
Methods: The Nimblegen sequence capture protocol began with 3-5 ug of high molecular weight genomic DNA from each animal. This DNA was used in each case to prepare a 454 LifeSciences Titanium shotgun sequencing library. From each of these libraries 15 ng of DNA was used in a ligation mediated (LM) PCR to bring the total amount of library DNA up to at ~ 3 ug. For hybridization with the SSc custom sequence capture arrays we combined: 1) 2 ug of the LM-PCR amplified library; 2) 100 ug of murine COT DNA (to hybridize with highly repetitive genomic sequences); 3) 650 picomoles of hybridization enhancing oligos; 4) 7.5 ul 2x sequence capture (SC) hybridization buffer; 5) 3 ul SC hybridization component A; and 6) 4.5 ul of each of the individual murine genomic libraries. This mixture was incubated at 47 °C for 72 hours. It was then added to 100 ul of Streptavidin Dynabeads to bind the biotin tags on the genome libraries. Incubation was at 47 °C for 45 min. The tubes were then placed in a Dynamag-2 device to bind the beads which were washed repeatedly to remove any genomic DNA that was not bound to the custom locus library array. The captured DNA was eluted and resuspended in water and then reamplified once more using LM PCR. A qPCR SYBR Green assay was used to determine the amount of enrichment by comparing samples before and after capture. This captured DNA was then subjected to 454 Titanium sequencing.

Initial assembly of the captured DNA into contigs was performed using the Newbler de novo assembler and then completeness of coverage and coverage depth were determined (see Table 1 for sequencing and alignment metrics). The contigs were then scaffolded against the latest curated mouse genome to ensure that all nonredundant sequences were captured and sequenced to a sufficient depth to ensure reliability of the data. For the four libraries we obtained between 116,000 and 157,000 reads of which 75-85% mapped to the target locus indicating that the capture beads had provided excellent targeted enrichment. Our average coverage levels ranged from 4.5 – 9.6X. Although as indicated above the number of probe bases was only

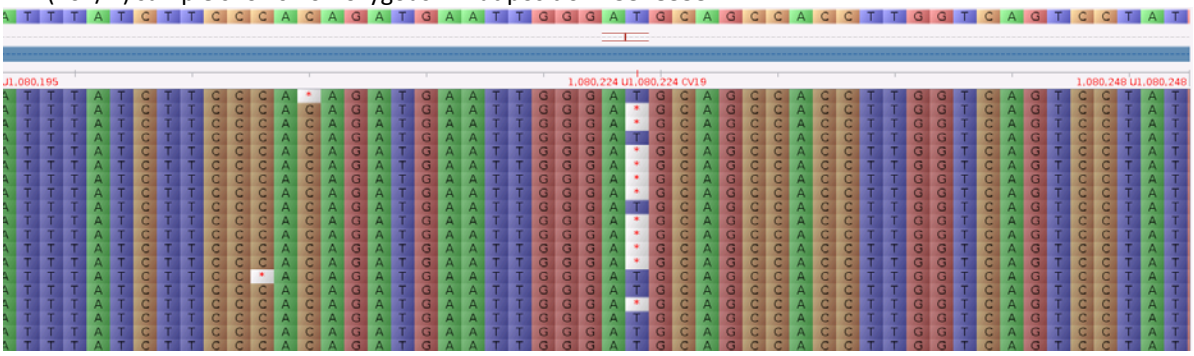
1.6M, we did recover an average of > 2.1 M bases due to the large fragment sizes of the captured DNA. Our average contig size was > 2 Kb. We used Mauve to identify allelic differences between the affected and

Table 1. SNPs found in candidate genes.

| Position | Corrected Pos | #CHROM | GWEIGHT | Gene | REF 2211 tsk2 | ALT 2211 tsk2 | QUAL tsk2 | REF 2214 tsk2 | ALT 2214 tsk2 | QUAL 2214 tsk2 | REF 2216 tsk2 | ALT 2216 tsk2 | QUAL 2216 tsk2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 45880257 | 1582128 | chr1 | 2 | WDR75 Exon | AC | CG | 2.9693 | A | C | 0.597642 | AC | CG | 19.8066 |
| 45432389 | 1134260 | chr1 | 2 | COL5A2 3'UTR | G | C | 183.141 | G | C | 158.412 | G | C | 319.07 |
| 45378353 | 1080224 | chr1 | 2 | COL3A1 Coding Exon | T | A | 1226.23 | T | A | 3032.56 | T | A | 289.282 |
| 45875728 | 1577599 | chr1 | 2 | WDR75 Coding Exon | T | C | 483.524 | T | C | 129.314 | T | C | 94.8358 |



WT (101/H) sample shows homozygous “T” at position 45378353



Tsk2/+ sample shows heterozygosity (T + A (indicated by *)) at this position.

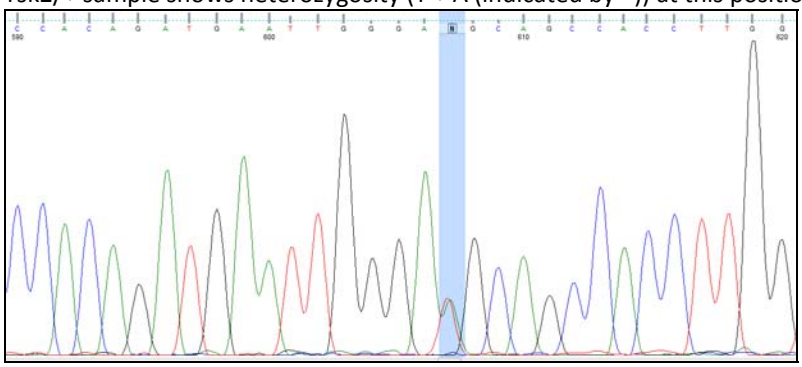


**Figure** 1. Individual sequence reads from 101/H (WT) sample (top) and Tsk2/+ sample (bottom). WT (101/H) sample shows homozygous “T” at position 45378353 Tsk2/+ sample shows heterozygosity (T + A (indicated by *) ) at this position. Bottom panel shows confirmation in PCR amplified genomic DNA from Tsk2/+ at Drexel of the SNP found by whole interval sequencing.

unaffected DNAs. The alignment is in progress, but based on a number of early results from excellent

candidate genes, we have narrowed in on several of these. As can be seen in Table 1 and **Figure** 1, the coding exon SNP in *Col3A1* is seen as heterozygous in Tsk2/+ samples, as expected.

We confirmed the global sequencing result at Drexel (**Figure** 1 bottom) and also evaluated the remaining SNPS by phototyping[1] . Of these, only the Col3A1 non-synonymous coding SNP was validated; two intronic SNPs in the GULP1 gene also distinguish Tsk2/+ from all other strains for which chr 1 genotyping is available. The Col3a1 SNP results in a Cys to Ser change in the PIIINP (N-terminal) cleavage product of the Col3a1, and thus is a target for our research in the immediate future.

2. *Expression of elastin*

We have nearly completed our study of the *expression of elastin* and its role in fibrosis. Elastin is a key component of the tight skin phenotype of Tsk2/+ mice. We had previously observed that abnormal collagen accumulation occurs with age in Tsk2/+ mice, but it does not occur until 10 weeks of age, fully 8 weeks after the development of the "tight skin" phenotype. While we observed transcripts of transforming growth factor (TGF)-β1 responsive genes with increased levels in Tsk2/+ skin, correlating with higher levels of TGF-β1 at 2 and 10 weeks of age, this didn't directly explain the very tight skin at two-three weeks of age.

**Figure 2. Top – A, Bottom – B**

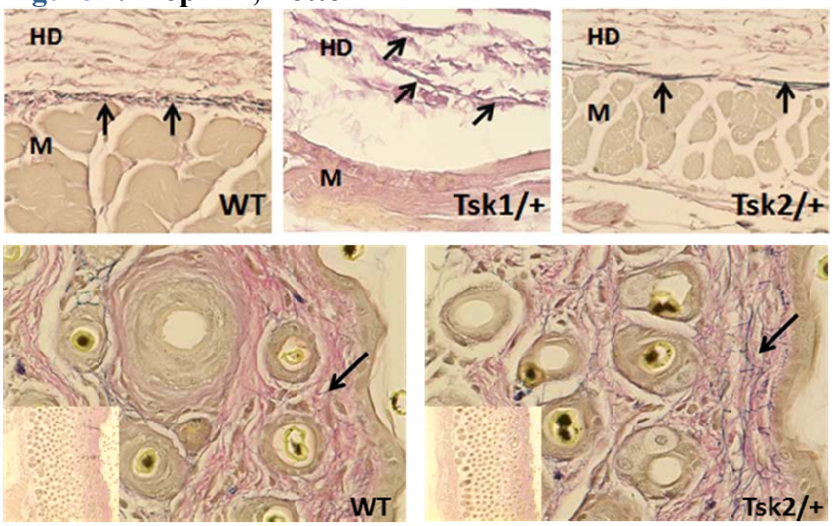



Fig 2C

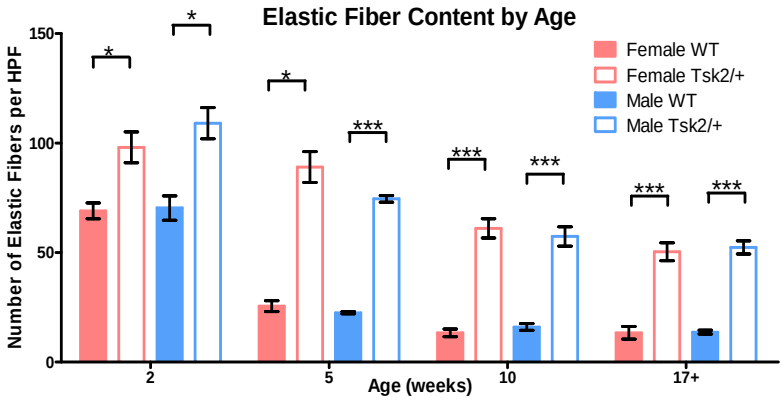


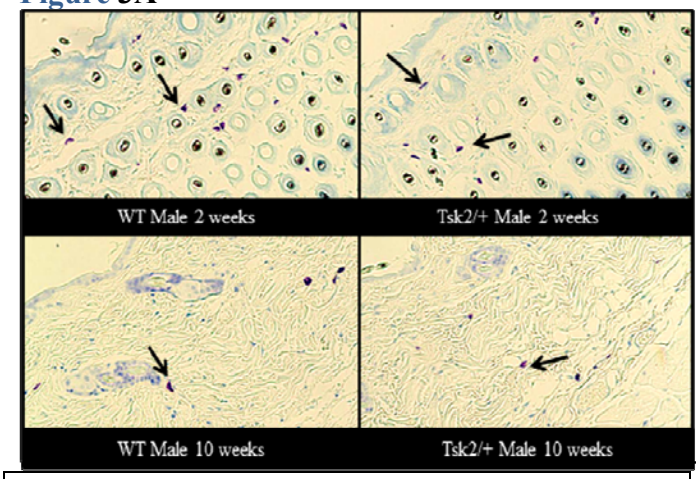

**Figure 2. Tsk2/+ mice have increased elastic fibers in skin.** Skin samples were stained with Weigert's Resorcin Fuchsin Stain and elastic fiber number per high powered field was calculated. **A,** Elastic fiber WT (left), Tsk1/+ (middle) and Tsk2/+ (right) male mice at 5 weeks of age (400X magnification) shown between the hypodermal muscle (M) and the hypodermal connective tissue (HD). Distinct elastin fibers are marked with arrows. **B,** Elastic fibers in the dermis from WT (left) and Tsk2/+ (right) female mice at 2 weeks of age. Images are shown at 400X magnification. **C,** Quantitative amount of elastic fibers per HPF over time. N=4-6 mice per group, 5-9 HPFs per slide. * p<0.05, **p<0.01, ***p<0.001.

We then found a highly significant increase in elastic fibers in 2-week-old Tsk2/+ mice that continues throughout adulthood. The timeline of disease development in the Tsk2/+ mouse shows that fibrosis is progressive, with novel elastic fiber changes occurring months before collagen accumulation. Tsk2/+ mice have increased dermal levels of TGF-β1 prior to the development of disease, suggesting that fibrosis is TGF-β1 driven and involves elastin, not collagen, in early stages.

Skin samples from Tsk2/+ and WT mice at 2, 4, 10, and 23 weeks of age were examined for other ECM anomalies, based on the increased expression of certain ECM genes early in life). As early as 2 weeks of age, Tsk2/+ skin had significantly more elastic fibers in the dermis compared to WT mice (**Figure** 2B, p<0.05). The increase in elastic fibers is maintained through all ages (**Figure** 2C). There was no difference in the elastic fiber layer beneath the panniculus carnosus in Tsk2/+ mice, unlike Tsk1/+ mice (**Figure** 2A), indicating another significant difference between the two models of disease. The difference between

Tsk2/+ and WT littermates in elastic fiber expression was one of the earliest and most reliable signatures of the Tsk2/+ phenotype and, unlike collagen deposition, predicted fibrosis and the tight skin phenotype seen in Tsk2/+ skin as early as the skin pinch and foot caliper assays.

**Figure 3A**



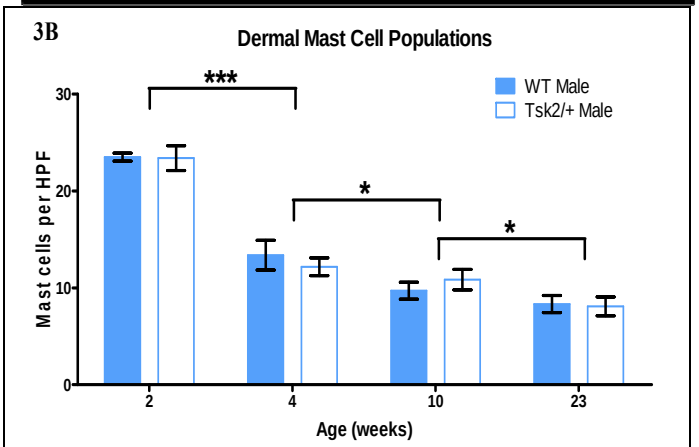




**Figure 3. Infiltrating mast cell populations in Tsk2/+ male mice are similar to WT male mice**. Skin sections were examined for mast cell numbers per high powered field (HPF). **A**, There is no difference in mast cell populations between Tsk2/+ and WT male mice at any age. **B**, There is a significant decrease in mast cell numbers with age. N=4-6 mice per group and 9 HPFs per slide. * p<0.05, ** p<0.01, *** p<0.001.

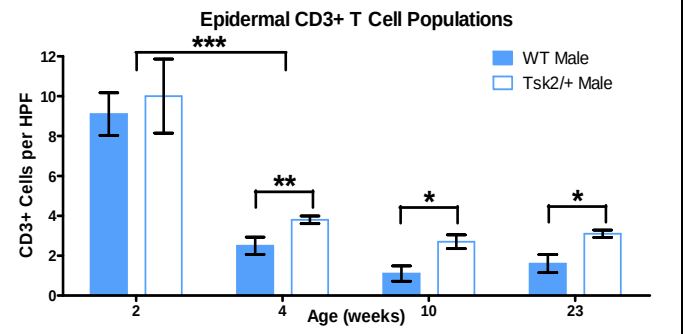



**Figure 4. Epidermal CD3+ T cell populations in Tsk2/+ male mice are slightly higher than in WT male mice**. Skin sections were examined for the number of CD3+ T cells per high powered field (HPF). There is a significant decrease in CD3+ T cell numbers between age 2 and 4 weeks of age. N=4-6 mice per group and 9 HPFs per slide. * p<0.05, ** p<0.01, *** p<0.001.

### 3. *Cellular infiltrates in Tsk2/+ mice*

In addition, we also found no substantial difference in the number of infiltrating cells in the skin of Tsk2/+ mice. Dermal mast cells were seen in equivalent numbers in Tsk2/+ and WT mice across all ages (**Figure** 3A) although all mice showed a significant decrease in mast cell numbers with age (~2-fold decrease between 2 and 4 weeks of age, p<0.0001). CD3+ T cell analysis showed a significant, yet small increase in the number of cells in the Tsk2/+ epidermis compared to WT littermates starting at 4 weeks of age (**Figure** 4). Importantly, there was no difference in CD3+ T cell number at 2 weeks of age. Like the mast cell decrease early in life, there was ~3-fold decrease in epidermal CD3+ T cells between 2 and 4 weeks of age (p<0.001).

We conclude that while there was no difference in dermal mast cell numbers, there is a very subtle increase in the number of infiltrating T cells in the skin of Tsk2/+ mice. The possible physiological impact of this infiltrate is questionable as it occurs after the tight skin phenotype is detectable. We have combined these results with material presented in the DOD grant proposal into a manuscript for the *Journal of Investigative Dermatology.*

### 4. *RNA gene profiling*

We have initiated the RNA gene profiling by microarray at Dartmouth. Dr. Whitfield's laboratory has focused on completion of Milestone 3, task 2 (months 4 – 12). To accomplish this milestone we have analyzed skin from both wt and Tsk2/+ mice at 4, 8, 12, and 20 weeks of age. We have also paid careful attention to the differences between males and females. We have analyzed the data from our DNA microarray hybridizations and found a clear time dependence on the gene expression in Tsk2/+. Analysis of the female mice at 4 weeks of age identified a specific gene expression signature of 405 genes (FDR < 10%) as determined by Significance Analysis of Microarrays (SAM; **Figure 5A**)[2]. We find that the majority of genes upregulated in Tsk2/+ mouse skin at 4 weeks of age map to the GO Biological processes of Cell adhesion and Cell morphogenesis (DAVID, Benjamini-corrected p < 0.05). Genes that shown increased expression include Col6a1, Col6a2,

Col5a1, Sparc and Thy1. Many of these genes are known targets of the profibrotic cytokine TGFbeta. Therefore, we have an initial indication that we will find high expression of TGFbeta responsive genes in mouse skin at 4 weeks of age. Therefore, we have begun to accomplish Task 3 under Milestone 3, to identify TGFbeta responsive gne signatures in the Tsk2 mouse (to be completed months 8 – 24). Genes with decreased expression at 4 wks were not enriched for any specific GO biological process or KEGG pathway. Analysis of differential gene expression between wt and Tsk2/+ mice at 8 weeks of age did not show any statistically significant differences. In contrast, analysis of differential gene expression between wt and Tsk2/+ mice at 12 weeks of age revealed striking differences in gene expression (**Figure 5B**). We find 1100 genes differentially expression (SAM, FDR<0.2%). Analysis of the GO biological processes show the dominant program among the upregulated genes is *M phase*, *cell cycle*, *mitosis*, *DNA packing* and *chromatin assembly*, suggesting the presence of proliferating cells [3, 4]. Genes included among these processes are the cell cycle regulators CCNB1, CCNA2, CDCA2, FOXM1 and PLK1. The genes with decreased expression at 12 weeks are enriched for the GO biological process of *Immune response* (DAVID, Benjamini-corrected p<0.05) and include genes IL18 receptor 1 (IL18r1), IL1beta, ccl7 and mst1. We currently do not have enough samples at 20 wks to perform this analysis. Additional samples will be added in the coming year to bring ensure statistical power to analyze the remaining time points.

These data are consistent with our earlier results (preliminary data for this grant) that showed Tsk2/+ resembles human SSc TGFbeta activated subset at 4 weeks, but not a 16 weeks.

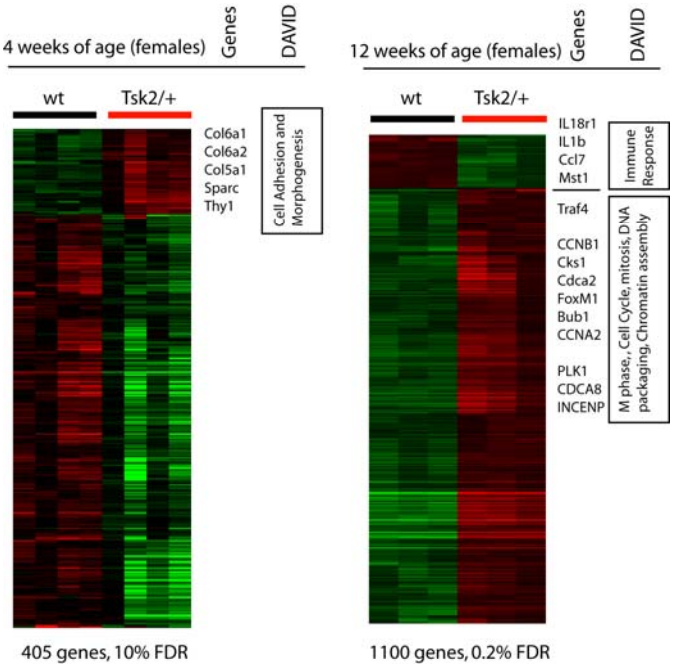



**Figure 5. Differential gene expression in skin between wt and Tsk2/+ female mice during development**. **A.** Mice at 4 weeks of age were analyzed for statistically significant differences in gene expression using significance analysis of microarrays (SAM) identifies 405 genes (FDR<10%). Gene expression is shown as a heat map with red indicating increased expression and green indicating decreased expression. Wt and Tsk2/+ are indicated. Select genes are indicated to the right of the heat map. GO biological process annotations enriched among these differentially expression genes as determined by DAVID are also indicated (Benjamini corrected p<0.05). **B.** Genes differentially expressed at 12 weeks of age as determined by SAM identifies 1100 genes (FDR<0.2%). Select genes and enriched GO Biological Processes are indicated.

### 5. *Functional analysis of Tsk2/+ candidate gene*

We have begun the analysis of Tsk2 candidate gene molecules at the protein level. We are still determining the best experimental approach to determine the potential difference in mechanotension of ECM containing collagen from Tsk2/+ vs. that containing collagen from WT. We have tried culturing the fibroblasts in flasks for 4 weeks to lay down matrix, however when we lysed the cells or trypsinized them to remove the cells, we also removed the collagen matrix. As we have previously studied the response of fibroblasts to collagen-coated dishes, we are approaching this problem by culturing Tsk2 or WT fibroblasts for 2 weeks to establish conditioned media that contains the collagen. Then 10 ml of that media containing secreted collagens was used to coat a new flask overnight. The media was removed and Tsk2/+ fibroblasts were seeded into flasks that had WT collagen, or WT fibroblasts were seeded into flasks that had Tsk2/+ collagen. Intriguingly, by this method we found that when Tsk2/+ fibroblasts were cultured on WT collagen, TGF-β1 transcripts were reduced; however, when either of the two WT fibroblast lines were cultured on collagen obtained from two

different Tsk2/+ mice, TGF-β1 transcripts were increased approximately 2-fold (Figure 6). We will be repeating this experiment with age- and sex-defined cell lines, but so far, WT cells make more TGFβ when cultured on Tsk2/+ matrices. This is exactly what would be expected if the collagens produced by Tsk2/+ were altered by the mutation in such a way as to affect interactions with normal non-mutant cells. This finding mimics the TGF-β1 signature that is seen in the Tsk2 mice and leads us to speculate that this signature may be derived from outside/inside signaling by the collagens.

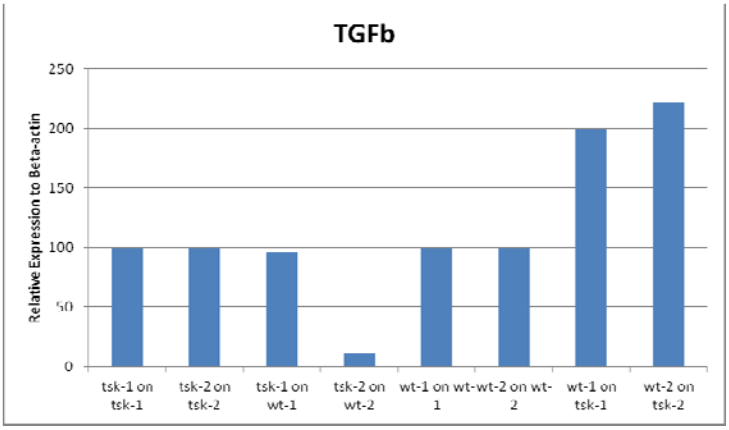



**Figure 6. TGF-β1 gene expression in fibroblasts cultured on normal and Tsk2 matrix.** Fibroblasts were seeded onto flasks coated with conditioned medium in a mix-and-match scheme, where cells from Tsk2/+ sample 1 were cultured on Tsk2/+ sample 1 or WT-sample 1 matrices. A duplicate experiment was performed with Tsk2/+ sample 2 cultured on Tsk2/+ sample 2 or WT-sample 2 matrices, WT-1 cells cultured on WT-1 or Tsk2/+ sample 1 matrices and WT-2 cells cultured on WT-2 and Tsk2/+ sample 2 matrices. Self combinations were normalized to 100.

**Reticular Fiber Stain.** We stained tissue sections from Tsk2/+ and wild-type skin for reticular fibers. Reticular fibers are comprised primarily of type III collagen and with the specific reticular fiber stain, they stain dark grey due to deposition of silver onto the fiber. In the dermis where there is less type III collagen, the collagen stains taupe/pale grey. Reticular fibers are found around the panniculus carnosus and we observed that in the Tsk2 mouse there was increased staining for reticular fibers as the mice age. We found that at 2.1 weeks of age a small staining difference was observed for Tsk2/+ in the amount of type III collagen between the muscle fibers. As the mice progressed to 10 weeks of age, the reticular fibers became less fine/fluffy and instead were thickened and the staining of the fibers was more pronounced. By 23 weeks of age, Tsk2 mice had strong staining of the collagen fibers which were substantially thickened than their wild-type littermates (Figure 7). In addition to the panniculus carnosus, we observed increased staining in the dermis for reticular fibers at age 2.1 weeks. The Tsk2/+ mice were found to have more densely packed taupe colored fibers and this was apparent from age 2.1 weeks. As the mice age through to 23 weeks, the dermis stained darker reflecting the increase in collagen III deposition. Note in the wild-type mice that the fibers are less densely packed than that observed in the Tsk2/+ mice.

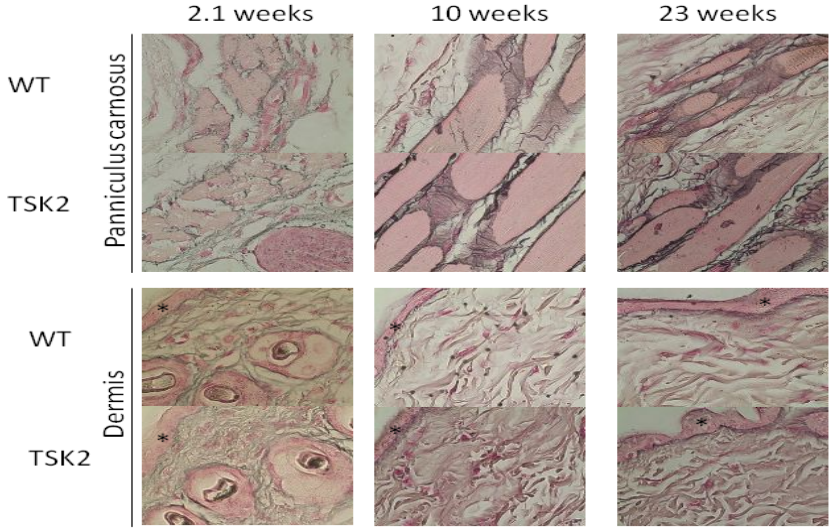



**Figure 7. Reticular fiber staining in Tsk2 and wild-type mice at 2.1, 10, and 23 weeks of age**. Sections were deparaffinized in 2 changes of xylene and 2 changes of ethanol, and then rehydrated. The sections were stained with 1% potassium permanganate, 3% potassium metabisulfite, ferric ammonium sulfate, and ammoniacal silver. The sections were then fixed in formalin and further stained with gold chloride and sodium thiosulfate, and then counterstained with nuclear fast red. Reticular fibers stain grey to dense black. The asterisk denotes the epidermis. All

**KEY RESEARCH ACCOMPLISHMENTS**

- We have started the 454 next generation sequencing of the mouse Tsk2/+ interval. Genomic DNA from two heterozygous Tsk2/+ mice and two WT littermates was sent to ASRI, and processed as described in the body of the progress report to yield four complete sequences.
- A number of single nucleotide polymorphisms have been discovered in these two new, unsequenced strains (101/H, the parental strain; and B6.tsk2, the B6 congenic strain bearing mutated 101/H DNA on chromosome 1 that contains the Tsk2/+ mutation). One of these is in *Col3A1*. This SNP and two others in intronic regions of the *Gulp1* gene have been verified.
- We have begun the breeding necessary for the genetic complementation test of *Col3a1* by breeding the Tsk2/+ line to BALB.Col3A1KO mice (heterozygotes as well). Results should be definitive and are expected soon.
- We have verified early results that the excess deposition of collagen matrix does not occur until well after the Tsk2/+ tight skin phenotype is evident. We have also verified that elastin is highly significantly increased in Tsk2/+ mice at the time the phenotype is present.
- We have new results suggesting that the collagen that is deposited in reticular fibers has a different appearance, even at early stages before frank fibrosis occurs in Tsk2/+ mice.
- Microarray profiling of Tsk2/+ mice at 4, 8, 12 and 20 weeks have been performed. We have identified genes that are differentially regulated at each time point. Tsk2/+ mice have a TGFβ1 signature that is seen in a global assessment of mRNA from skin in a carefully controlled study using littermates (Tsk2/+ and WT) at timed stages and stratified by sex.

**REPORTABLE OUTCOMES**

***Manuscripts, abstracts, presentations***

Manuscripts:

Kristen B. Long, Carol M. Artlett, and Elizabeth P. Blankenhorn. Tight Skin 2 Mice have Increased TGF-β1 Levels Prior to Disease Development leading to Excessive Elastic Fibers in Skin. Manuscript to be submitted to *J. Invest. Dermatol*., 2012

Presentations:

C.M. Burgwin, K.B. Long, Zhenghui Li*, C.M. Artlett, M. Whitfield*, and E.P. Blankenhorn. "Mapping of the Mutation in the Tight Skin 2 model of systemic sclerosis" Department of Microbiology and Immunology, Drexel Univ. College of Medicine, Philadelphia, PA. *Dartmouth Medical School, Hanover, NH, Institute for Molecular Medicine and Infectious Disease International Symposium, Philadelphia, PA, June 2012

Burgwin, C.M., K.B. Long, Z. Li., C.M. Artlett, M.L. Whitfield* and E.P. Blankenhorn. "Mapping of the mutation in the Tight Skin 2 mouse model of systemic sclerosis." Department of Microbiology and Immunology, Drexel Univ. College of Medicine, Philadelphia, PA. *Dartmouth Medical School, Hanover, NH, 21st Annual Infection and Immunity Forum, Eastern PA Branch of ASM, Drexel University College of Medicine, Philadelphia, Pennsylvania, June 2012.

John, A.K., K.B. Long, L. Cort and E.P. Blankenhorn. "Fibroblast investigations suggest that increased collagen production is cell-autonomous in the Tsk2/+ mouse model of scleroderma." Drexel University College of Medicine, Discovery Day Research Symposium, Philadelphia, Pennsylvania, October 2011.

Long, K.B., C.M. Burgwin, C.M. Artlett and E.P. Blankenhorn. "Examination of pre-fibrotic and fibrotic changes in Tsk2/+ mice uncovers a novel time line of disease development and a possible new phenotype in human

disease." Platform Oral Presentation, Discovery Day Research Symposium, Drexel University College of Medicine, Philadelphia, Pennsylvania, October 2011.

Long, K.B., C.M. Burgwin, C.M. Artlett and E.P. Blankenhorn. "Examination of pre-fibrotic and fibrotic disease in Tsk2/+ mice shows early ECM changes are attributable to elastin dysregulation." 12th International Workshop on Scleroderma Research, Cambridge, UK, July 2011.

***Degrees obtained that are supported by this award***

Kristen B. Long successfully defended her PhD thesis, entitled "Examination of a model of systemic sclerosis, the Tight Skin 2 mouse: before, during and after fibrotic disease" in April, 2012. She was awarded her PhD by Drexel University College of Medicine in May 2012. Her work was directly supported by this grant.

***Development of cell lines, tissue or serum repositories***

We have developed a large number of Tsk2/+ and WT littermate fibroblast cell lines from mice of various ages and both sexes.

**CONCLUSION**

We have shown a clear time dependence on the gene expression in the skin of the Tsk2/+ mice. The mouse most resembles human SSc at a narrowly defined time point (4 wks of age) which means studies that use this model as a surrogate for human SSc, must use specific time points in their analysis. We have pinpointed at least one candidate gene in the interval for Tsk2/+ and have confirmed the sequence difference between Tsk2/+ and the parent strain, 101/H[5]. We present preliminary results on the expression of TGFβ mRNA from cells cultured on ECM from Tsk2/+ and WT littermates that suggest a mechanism for the up-regulation of TGFβ seen in the mutant strain. We show that elastin content in the skin, known to be controlled by TGFβ[6] and possibly up-regulated in SSc[7], is the earliest indicator of tight-skin in the tissue.

**REFERENCES**

1. Bunce, M. *et al.* Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue antigens* **46**, 355-367 (1995).
2. Tusher, V.G., Tibshirani, R. & Chu, G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc.Natl.Acad.Sci.U.S.A* **98**, 5116-5121 (2001).
3. Whitfield, M.L. *et al.* Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Mol.Biol.Cell* **13**, 1977-2000 (2002).
4. Whitfield, M.L., George, L.K., Grant, G.D. & Perou, C.M. Common markers of proliferation. *Nat Rev Cancer* **6**, 99-106 (2006).
5. Peters, J. & Ball, S.T. Tight skin-2 (Tsk2). *Mouse News Letter* **74**, 91-92 (1986).
6. Oleggini, R., Gastaldo, N. & Di Donato, A. Regulation of elastin promoter by lysyl oxidase and growth factors: cross control of lysyl oxidase on TGF-beta1 effects. *Matrix biology : journal of the International Society for Matrix Biology* **26**, 494-505 (2007).
7. Quaglino, D., Jr. *et al.* Connective tissue in skin biopsies from patients suffering systemic sclerosis. *J Submicrosc Cytol Pathol* **28**, 287-296 (1996).

**APPENDIX**

Manuscript by Long, Artlett, and Blankenhorn follows this page

# Tight Skin 2 Mice have Increased TGF-β1 Levels Prior to Disease Development leading to Excessive Elastic Fibers in Skin

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Abbreviations:

Full-length article

Running Title: TGF-β1 related abnormalities in Tsk2/+ mice

**ABSTRACT**

The Tsk2/+ mouse model of systemic sclerosis (SSc) has many features of the human disease including tight skin, fibrosis, extracellular matrix abnormalities, and occurrence of antinuclear antibodies (ANA). This model was used to understand disease progression and to evaluate early pathological changes essential for fibrosis and disease development that are difficult to study in patients with SSc.

Abnormal collagen accumulation occurs with age in Tsk2/+ mice, but it does not occur until 10 weeks of age, fully 8 weeks after the development of the "tight skin" phenotype. Transcripts of transforming growth factor (TGF)-β1 responsive genes showed increased levels in Tsk2/+ skin, correlating with higher levels of TGF-β1 at 2 and 10 weeks of age. ANA production is a late phenotype and is not different between Tsk2/+ and WT littermates. However, we observed a highly significant increase in elastic fibers in 2-week-old Tsk2/+ mice that continues throughout adulthood.

The timeline of disease development in the Tsk2/+ mouse shows that fibrosis is progressive, with novel elastic fiber changes occurring months before collagen accumulation. Tsk2/+ mice have increased dermal levels of TGF-β1 prior to the development of disease, suggesting that fibrosis is TGF-β1 driven and involves elastin, not collagen, in early stages.

## INTRODUCTION

Systemic Sclerosis (SSc) is a polygenic, autoimmune disorder of unknown etiology, characterized by the excess accumulation of the extracellular matrix (ECM), vascular alterations, and presence of antinuclear antibodies (ANA) [1, 2]. SSc is classified into two major subsets based on the proportion of skin involvement. Limited cutaneous systemic sclerosis (lcSSc) involves dermal thickening of skin below the elbows and knees with or without facial involvement, while diffuse cutaneous systemic sclerosis (dcSSc) manifests as dermal thickening of the skin of the extremities, face, and trunk, as well as involvement of at least one internal organ, including the lungs, kidneys, heart, esophagus, and gastrointestinal tract [3]. Currently, the 10-year survival rate for patients suffering from lcSSc ranges from 69-90%, while the rate for patients suffering from dcSSc ranges from 53-70% [4-6]. There is no cure for SSc and only palliative treatment is available.

There are several animal models of disease that have provided insights into SSc pathology. However, a model exhibiting all signs of SSc has not been established [7, 8]. There are two main genetic mouse models of disease, the Tight Skin (Tsk) 1 [9-11] mouse and the Tsk2 mouse [12, 13]. Each mouse strain bears a different homozygous lethal mutation that requires mice to be bred and evaluated as heterozygotes (e.g., Tsk1/+ and Tsk2/+). The cause of fibrosis in Tsk1/+ mice is a mutation in the fibrillin1 gene *(Fbn1*) on chromosome 2 [14], whereas the gene causing scleroderma-like signs in Tsk2/+ mice, located on chromosome 1 at a position between 42.3-52.3 megabases [15], is unknown. Tsk1/+ mice develop excess skin fibrosis; however, the collagen accumulation occurs in the hypodermis [9], in contrast to the dermal collagen accumulation in SSc. Lung disease in Tsk1/+ mice mimics an emphysema-like disease [11], whereas involved lung tissue in patients with SSc is characterized by fibrosis often with vasculopathy such as pulmonary hypertension. Additionally, the development of ANA is limited in Tsk1/+ mice whereas greater than 90% of SSc patients produce ANA [1].

In contrast, Tsk2/+ mice have increased collagen and ECM changes in the dermis, like that seen in SSc. The timing of these changes is controversial. Studies by Christner et al. have shown a marked increase in collagen accumulation in the dermis of 10 day old Tsk2/+ mice, which was still present at 7-8 months of age compared to wild-type (WT) littermates; in addition, they observed an infiltrate of mononuclear inflammatory cells in

adult Tsk2/+ mice [12]. A subsequent reported failed to confirm this finding [16]. Christner et al. also showed increased transcription of Col1a1 and Col3a1 and an up-regulation of collagen production in fibroblasts isolated from Tsk2/+ mice of unknown sex and age [17]. Barisic-Dujmovic et al. showed that cultured Tsk2/+ fibroblasts had increased expression of a collagen I–promoter–driven GFP reporter compared to control fibroblasts, but this finding does not address the timing of up-regulation of collagen *in vivo* [16]. This group also noted that Tsk2/+ mice have a reduction in thickness of the adipose layer in the dermis, a significant reduction in body size and mass, and normal lung morphology at 2-3 months of age [16]. Finally, studies by Gentiletti et al. revealed that aged Tsk2/+ on a C3HxC57Bl/6J background produce numerous ANAs [18] that are also seen in human SSc.

In the present study, we examined disease pathology and progression over time in Tsk2/+ male and female mice. We report a novel timeline of events in Tsk2/+ disease development and show that the signs of fibrotic disease are progressive, starting from two weeks of age. Tsk2/+ mice were found to have increased levels of TGF-β1 and excessive elastic fiber accumulation when they are only 2 weeks old, far earlier than when dermal fibrosis appears at 10 weeks of age. This suggests that elastin, *not collagen*, is responsible for the "tight skin" phenotype at weaning age and is TGF-β1 dependent. In addition, we demonstrate that ANAs are present in Tsk2/+ mice only *after* disease is well established.

**RESULTS**

**Footpad thickness predicts the tight skin phenotype in Tsk2/+**. The tight skin phenotype in Tsk2/+ mice is evident upon pinching the interscapular skin of young mice as early as 2 weeks of age [12], but this assessment is variable and subjective [9]. To provide a more quantitative assay for the Tsk2/+ trait, we assessed the thickness of mouse footpads corrected for body weight. Significant weight differences were observed between male and female mice ($p<0.0001$), as well as between same-sex Tsk2/+ and WT mice ($p<0.001$) (Figure 1A), as expected from previous observations by Barisic-Dujmovic et al. [16]. Footpad thicknesses were highly significantly associated with inheritance of the *Tsk2* gene, with low variance, and excellent reproducibility (Figure 1B and 1C). At 4-6 weeks of age, Tsk2/+ male and female mice have significantly thicker footpads than WT mice ($p<0.0001$), with no sex difference (Figure 1B). At 10-12 weeks of age (Figure 1C) and $\geq$17 weeks of age (data not shown), Tsk2/+ males had significantly thicker footpads than Tsk2/+ females ($p=0.0004$ and $p<0.0001$ respectively). At all stages, Tsk2/+ mice had thicker footpads than WT mice of the same sex ($p<0.0001$).

**Protein content of footpads shows collagen accumulation occurs with age in Tsk2/+ mice.** To assess total collagen accumulation in the footpad with age, we used the hydroxyproline assay. As noted above, Tsk2/+ mice weigh significantly less than their WT counterparts. Because of this body size difference (Reference [16] and Figure 1A), individual footpads were weighed from Tsk2/+ and WT mice, and it was determined that Tsk2/+ mice had significantly smaller footpads ($p<0.004$, data not shown) although they are thicker (Figure 1B and 1C) than those of WT mice; a difference that was apparent at 2 weeks of age. Assessment of hydroxyproline content, normalized to the weight of the footpad, revealed no difference between Tsk2/+ and WT mice or between male and female mice at 2 or 4 weeks of age. However, by 10 weeks of age there was a significant increase in the amount of footpad collagen content in Tsk2/+ mice that continued into adulthood (Figure 1D). Thus, the increase in collagen content of the footpad occurs over time.

**Dermal collagen accumulation occurs with age in Tsk2/+ mice**. Skin samples from Tsk2/+ and WT mice from three age ranges were analyzed for total collagen content by Masson's Trichrome staining (Figure 2A). At

4-5 weeks of age, there was no significant difference in skin thickness between Tsk2/+ and WT mice, but there was a sex difference with male mice having increased collagen thickness compared to females ($p<0.0001$, data not shown). At 10 weeks of age, the increase in collagen in Tsk2/+ mice compared to WT mice was significant in both males and females ($p<0.01$ and $p<0.05$, respectively) and this significant difference continued to 17+ weeks of age ($p<0.001$ and $p<0.01$, respectively) (Figure 2B). There is also compaction and loss of dermal adipose tissue in Tsk2/+ males at 17+ weeks of age, as observed previously [16].

Skin samples across the same age ranges were analyzed for total collagen content by the hydroxyproline assay. At the younger ages (2-4 weeks old) stratified by sex there was no difference between Tsk2/+ and WT mice for hydroxyproline content of the skin. However, there was an increase in hydroxyproline in skin at 10 and 23 weeks of age, with Tsk2/+ mice having significantly more hydroxyproline in skin than WT mice (Figure 2C). At all ages except 2 weeks, male skin had significantly more hydroxyproline than female skin ($p<0.05$ at 4 and 10 weeks and $p<0.01$ at 24 weeks). The hydroxyproline assay therefore confirmed the Trichrome assay and the histological findings of fibrosis in the skin of Tsk2/+ mice at the later ages. Thus, the increase in collagen content of the skin of Tsk2/+ mice, as measured by two independent assays, occurs after the appearance of the tight skin phenotype.

**Histological assessment of cells in the pre-fibrotic and fibrotic skin of Tsk2/+ mice.** Another factor that could explain the unusual skin phenotype might be a cellular infiltrate in the Tsk2/+ mice, with concomitant inflammation. To test this possibility, skin samples from 2, 4, 10 and 23 weeks of age were examined for presence of infiltrating cells. Weigert's Hematoxylin staining, part of Masson's Trichrome stain, revealed no conclusive mononuclear cell difference at any age (data not shown). Additionally, toluidine blue staining showed that mast cells decrease with age, but do not differ between Tsk2/+ mice and WT littermates at any age tested (Figure 3A). Immunofluoroscence (IF) staining to reveal CD3+ T cells showed a significant but small increase in CD3+ T cells in the epidermis and surrounding the hair follicle in Tsk2/+ mice at 5, 10 and 23 weeks of age but not at 2 weeks of age (Figure 3B).

**Tsk/2+ mice have increased levels of mRNA for collagens, ECM proteins and transforming growth factor-beta1 (TGF-β1) signatures.** Messenger RNA expression levels were assessed from skin of Tsk2/+ and

WT mice at 2, 4, 10, 18 and 30 weeks of age using qRT-PCR analysis. There were no major differences between Tsk2/+ and WT adult mice after 30 weeks of age for these RNA transcripts (data not shown). We found a significantly higher abundance of mRNA for collagens, TGF-β1-induced genes, and ECM proteins in Tsk2/+ female mice at 4 and 18 weeks of age, and in Tsk2/+ male mice at 2 and 10 weeks of age (Table 1).

**Tsk2/+ mice have increased levels of total TGF-β1 in skin, yet no difference in the number of α-Smooth Muscle Actin (αSMA) positive cells.** Skin samples at varying ages from Tsk2/+ and WT mice were examined for the presence of total TGF-β1 and αSMA, a marker of myofibroblasts, using IF. Staining revealed that total TGF-β1 levels were increased in Tsk2/+ skin at 2 and 10 weeks of age (males only, Figure 4A). IF staining for αSMA positive cells showed that αSMA positive cells decrease with age, but do not differ between Tsk2/+ mice and WT littermates at any age tested (Figure 4B).

**Tsk2/+ mice have excess elastic fibers in the dermis.** Because the tight skin phenotype is readily observed in Tsk2/+ mice at 2-3 weeks of age, and yet there is not an excess of collagen at this stage, nor is there a remarkable cellular infiltrate, we sought another explanation for their skin tightness. Skin samples from Tsk2/+ and WT mice at 2, 4, 10, and 23 weeks of age were examined for other ECM anomalies, based on the increased expression of certain ECM genes early in life (Table 1). As early as 2 weeks of age, Tsk2/+ skin had significantly more elastic fibers in the dermis compared to WT mice (Figure 5B, $p<0.05$). The increase in elastic fibers is maintained through all ages (Figure 5C). There was no difference in the elastic fiber layer beneath the panniculus carnosus in Tsk2/+ mice, unlike Tsk1/+ mice (Figure 5A, and Reference [19]).

**Tsk2/+ mice produce ANAs *after* disease signs are initiated.** There was no difference in the occurrence of ANA production between BC3 to BC5 generations of C57Bl/6J (B6).Tsk2/+ mice or their WT littermates (Figure 5A), although female mice did produce ANAs in higher proportions compared to male mice ($p=0.02$). However, all ANA production occurred after 14 weeks of age (data not shown).

**DISCUSSION**

Previous studies have shown that Tsk2/+ mice develop excess skin fibrosis, demonstrate ECM anomalies, and produce ANA [12, 18]; however, the progression of such disease signs during aging have never been examined and a causative role for collagen accumulation in the fibrotic trait has not been proven. In order to determine disease progression, we needed an effective and efficient way to measure disease severity. In order to eliminate the variability inherent in the skin pinch method [9], we quantified disease severity by measuring the footpad thickness. Tsk2/+ mice have noticeably tighter scapular skin than littermates at 2 weeks of age, and thicker footpads than WT mice starting at 3 weeks of age. Tsk2/+ males have thicker footpads than Tsk2/+ females at 10-12 weeks of age (Figure 1B, 1C). Footpad collagen content was not increased in Tsk2/+ mice until 10 and 23 weeks of age (Figure 1D). Therefore, the collagen levels do not have the same correlative value with disease as the caliper assay of footpads, and the initial increase in footpad thickness is caused by some anomaly other than collagen.

We also found no substantial difference in the number of infiltrating cells in the skin of Tsk2/+ mice. Dermal mast cells were seen in equivalent numbers in Tsk2/+ and WT mice across all ages (Figure 3A) although all mice showed a significant decrease in mast cell numbers with age (~2-fold decrease between 2 and 4 weeks of age, $p<0.0001$). CD3+ T cell analysis showed a significant, yet small increase in the number of cells in the Tsk2/+ epidermis compared to WT littermates starting at 4 weeks of age (Figure 3B). Importantly, there was no difference in CD3+ T cell number at 2 weeks of age. Like the mast cell decrease early in life, there was ~3-fold decrease in epidermal CD3+ T cells between 2 and 4 weeks of age ($p<0.001$). We conclude that there is a very subtle increase in the number of infiltrating T cells in the skin of Tsk2/+ mice, but the possible physiological impact of this infiltrate is questionable as it occurs after the tight skin phenotype is detectable.

Two independent methods to determine total collagen protein revealed that the increase in collagen protein accumulation in Tsk2/+ mice does not occur until 10-12 weeks of age (Figure 2), well after the skin and footpad phenotypes. There was a statistically significant higher abundance of Col5a2 mRNA in male skin samples at 2 weeks of age, and Col1a1, Col3a1 and Col5a2 at 10 weeks of age (Table 1), but this difference varied by sex: a

higher abundance of Col1a1, Col3a1 and Col5a2 mRNA in female Tsk2/+ mice only at 4 and 18 weeks of age (Table 1). Thus, few of the expected culprits discriminate the skin of Tsk2/+ at a time when the tight skin phenotype is apparent.

We compared mRNA from Tsk2/+ skin with that of WT mice for other possible triggers of disease. Gene expression associated with elastic fibers, such as elastin, fibrillin-1, fibulin-2 and fibulin-5, was increased in Tsk2/+ females at 4 and 18 weeks of age and in Tsk2/+ males beginning at 2 weeks (Table 1). The elastin gene is increased nearly 13-fold in female Tsk2/+ relative to WT females at 4 weeks, 2.7-fold in males at 2 weeks. Although the TGF-β1 mRNA expression level was never significantly different between Tsk2/+ and WT mice, there was a significant up-regulation of TGF-β1 targets [20] (Acta2, the gene for smooth muscle actin, cartilage oligomeric matrix protein (Comp) and Sparc, commonly referred to as osteonectin), and IF showed the elevated presence of TGF-β1 protein in male Tsk2/+ mouse skin as early as two weeks. Surprisingly, however, there was no difference in the number of αSMA-positive cells by IF at any age, even though the mRNA is seen in excess in Tsk2/+ mice. Overall, because of the increase in TGF-β1 levels and the increase in the gene expression of its targets at an age that is 6-8 weeks prior to fibrosis, we suggest that collagen accumulation in Tsk2/+ is TGF-β1 dependent, but that collagen protein accumulation itself does not precede the tight skin seen in these mice.

We therefore examined the elastic fibers present in the dermis of Tsk2/+ for three reasons: (1) we observed increased mRNA expression levels of proteins essential for elastic fiber assembly in the skin of Tsk2/+ mice (Table 1), (2) it has been suggested that patients with SSc have alterations in elastic fibers [21, 22] and (3) studies in the Tsk1/+ mouse have shown that this tight skin phenotype at weaning age is concomitant with dysregulation of elastic fibers beneath the panniculus carnosus in the hypodermis [19]. Two-week old Tsk2/+ mice (an age when there is no collagen accumulation difference) exhibit ~2-fold more elastic fiber protein in the dermis than WT littermates (Figure 5B, C). The excess elastic fibers were seen in the same dermal location as in human lesional sections [21, 22]. This difference is significant across all ages of Tsk2/+ mice, suggesting that elastin dysregulation precedes other signs of fibrosis and accounts for the initial tight skin phenotype at weaning age. There was no difference in elastic fibers *beneath* the panniculus carnosus in the hypodermis, as seen in Tsk1/+ mice (Figure 5A, and Reference [19]), indicating another significant difference between the two models

of disease. The difference between Tsk2/+ and WT littermates in elastic fiber expression was one of the earliest and most reliable signatures of the Tsk2/+ phenotype and, unlike collagen deposition, predicted fibrosis and the tight skin phenotype seen in Tsk2/+ skin as early as the skin pinch and foot caliper assays.

Because greater than 90% of patients with SSc produce ANA [1], we questioned whether ANA production drives disease or if it is just a result of disease development. Our antibody study revealed that there were no differences in total ANA production between Tsk2/+ and WT mice (data not shown). This finding disagrees with data previously published by Gentiletti, et al. 2005, who reported that Tsk2/+ mice have significantly more antibody production than WT mice at 4-6 months, 7-12 months and 13-24 months of age. The differences in findings could be due to age of the mice and the background of the WT mice, as their control mice were CAST/ei and C3H/HeJ [18], whereas our control mice are littermates on the C57Bl/6J background. Because ANA production is seen *after* the initiation of disease progression, we argue that antibody production is *marker* of disease progression and severity and not the *cause* of disease.

This study allowed for the development of a novel time line of disease progression in Tsk2/+ mice, leading to the investigation of changes occurring in the ECM pre-fibrosis. We have established that dermal elastic fiber accumulation occurs weeks prior to dermal collagen accumulation, and ANA production occurs weeks after fibrosis. Concomitant with increased elastic fiber accumulation was increased levels of dermal TGF-β1, suggesting that disease development in Tsk2/+ mice is TGF-β1 driven. This presents a novel pathway of disease development in Tsk2/+ mice.

## MATERIAL AND METHODS

**Mice:** Breeding pairs of Tsk2/+ mice were obtained from Dr. Paul Christner (Thomas Jefferson University, Philadelphia PA) with permission from the MRC Radiobiology Unit Laboratory, Chilton, UK and were housed at Drexel University College of Medicine. The Tsk2/+ mice were bred initially onto a hybrid (C3H x B6) background. Serial backcrossing (BC) of Tsk2/+ mice to B6 mice was initiated, and mice used in this study ranged from BC3 to BC5. For each experiment, age- and sex-matched WT littermates were used as controls. All studies and procedures were approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine.

**Footpad measurement of disease severity:** The mice were restrained with the left leg extended and a Starrett Catch and Release Analog Caliper was placed in the middle of the footpad and the resulting thickness of the pad was recorded. Mice between 4-24 weeks of age were weighed and their footpad thickness measured weekly. Because footpad size and thickness increases with age and body size, footpad thickness was binned by body weight at three different age ranges: 4-6, 10-12, and 17+ weeks of age.

**Determination of hydroxyproline content in skin and footpad tissue:** Mice at 2, 4, 5-6, 10-12, and 18-23 weeks of age were sacrificed and the lower dorsal fur was removed by shaving. The skin was cleaned with iodine and 70% alcohol. Two 8 mm punches were excised from each piece of skin, snap-frozen, weighed, and stored at -80 $^{o}$C. Footpads were cut just above the pad and processed in the same manner. Protein was isolated for hydroxyproline assays, adapted from the UCSF protocol (http://baygenomics.ucsf.edu/protocols/Bleomycin_induced_pulmonary.pdf). Skin punches were homogenized in water and a 2:1 chloroform/methanol mixture was added to remove lipids. The homogenate mixture was shaken then centrifuged to separate the phases. The organic and aqueous phases were discarded, and the interphase was resuspended in water, and 50% TCA was added and the protein collected by centrifugation. The protein pellet was hydrolyzed with 12 M HCl for 16 hours at 110$^{o}$C under vacuum, and then reconstituted in water. Chloramine T solution was added to each sample, followed by the addition of Ehrlich's solution. The

optical density was read at 550 nm in triplicate and the hydroxyproline content was determined against a standard curve of hydroxyproline (Sigma-Aldrich, St. Louis, MO).

**Histological assessment of dermal fibrosis and ECM abnormalities:** Skin samples were excised, placed in 4% paraformaldehyde and paraffin embedded. Sections were stained with Masson's Trichrome to allow for visualization of collagen [23]. Digital images were obtained using a 100X final magnification with a Nikon Eclipse 80*i* microscope. The high powered field (HPF) of each section was standardized for the photographic area (1200x1200 pixels) allowing a direct comparison between images. The collagen area per image was traced and measured using Image J software (http://rsbweb.nih.gov/ij/) and the percent of collagen per standardized HPF was calculated.

Elastic fibers in the skin were visualized using Weigert's Resorcin Fuchsin Stain according to Vaalamo et al., [24]. Briefly, sections were stained with Weigert's Iron Hematoxylin solution, followed by Resorcin Fuchsin solution (Electron Microscopy Sciences, Hatfield, PA) for 9 hours. Sections were counterstained in Van Geison's Solution (Electron Microscopy Sciences) for 2 minutes. The number of elastic fibers per HPF was counted.

**Histological assessment of infiltrating cells, αSMA positive cells and TGF-β1:** Samples were stained with toluidine blue (http://www.ihcworld.com/_protocols/special_stains/toluidine _blue.htm) to visualize mast cells. Paraffin embedded sections were deparaffinized, hydrated and stained with 0.02% toluidine blue O/1% NaCl, pH 2.4 for 4 minutes, washed briefly, and permanently mounted. Mast cells were counted per standardized HPF at 100 X magnification. Immunofluorescence (IF) was used to visualize CD3+ T cells, αSMA cells and TGF-β1. Briefly, antigens were unmasked using 10 mM citrate buffer and sections blocked in 5% goat serum. Rabbit anti-CD3 polyclonal antibody (Abcam, Cambridge, MA) was used at a dilution of 1:250, rabbit anti-TGF-β1 polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was used at a dilution of 1:100, and mouse anti-αSMA monoclonal antibody (Lab Vision IHC System Solutions, Kalamazoo, MI) was used at a dilution of 1:5,000 in blocking buffer and incubated at 4 $^{o}$C overnight. Cy3-conjugated goat anti-rabbit IgG (JacksonImmuno Research Laboratories, Inc, West Grove, PA) was used at a dilution of 1:500 to visualize CD3+ cells and TGF-β1. Cy3-conjugated goat anti-mouse IgG (JacksonImmuno Research Laboratories) was

used at a dilution of 1:10,000 to visualize αSMA. DAPI (Invitrogen) was used to visualize nuclei. IF images were taken at 400X magnification.

**RNA isolation and real-time PCR**: Skin was snap-frozen and homogenized in TRIzol (Invitrogen, Carlsbad, CA). A standard protocol for RNA extraction was used, following Invitrogen's directions. Immediately after redissolving RNA in water, DNA was removed and RNA was purified using an RNeasy Mini Kit (Qiagen Sciences, MD). cDNAs were synthesized from 1.0 μg of total RNA using MultiScribe Reverse Transcriptase and random hexamers (Applied Biosystems, Foster City, CA). Primers for quantitative real-time PCR (qRT-PCR) were designed using the Primer 3 online program (http://frodo.wi.mit.edu) and synthesized by Integrated DNA Technologies. Relative quantification of all products was measured using SYBR Green chemistry (Applied Biosystems, Foster City, CA). Expression was normalized to actin and relative expression of each gene was calculated using $\Delta C_t$ formula. The fold increase or decrease in Tsk2/+ skin was calculated as a ratio over the expression in WT controls.

**IF assay for ANA:** Plasma was obtained by submandibular bleeding of Tsk2/+ and WT mice between 12-26 weeks of age. The plasma was diluted 1:20 in PBS and applied to slides pre-coated with HEp-2 cells (Bio-Rad, Redmond, WA), washed, then FITC-conjugated goat anti-mouse IgG (JacksonImmuno Research Laboratories) diluted 1:100 was applied [18]. IF images were taken at 100X, 200X and 400X magnification.

**Statistics:** Significance of the biological differences between Tsk2/+ and WT littermates was calculated using non-linear regression (Figure 1A), linear regression (Figure 1B, 1C) and the student's t-test (all other figures), with $p<0.05$ taken as significant.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare and have not received financial support from commercial sources.

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## REFERENCES


1. Okano, Y., *Antinuclear antibody in systemic sclerosis (scleroderma).* Rheum Dis Clin North Am, 1996. **22**(4): p. 709-35.
2. LeRoy, E.C., et al., *Scleroderma (systemic sclerosis): classification, subsets and pathogenesis.* J Rheumatol, 1988. **15**(2): p. 202-5.
3. Khanna, D. and C.P. Denton, *Evidence-based management of rapidly progressing systemic sclerosis.* Best Pract Res Clin Rheumatol, 2010. **24**(3): p. 387-400.
4. Steen, V.D., *Autoantibodies in systemic sclerosis.* Semin Arthritis Rheum, 2005. **35**(1): p. 35-42.
5. Scussel-Lonzetti, L., et al., *Predicting mortality in systemic sclerosis: analysis of a cohort of 309 French Canadian patients with emphasis on features at diagnosis as predictive factors for survival.* Medicine (Baltimore), 2002. **81**(2): p. 154-67.
6. Ferri, C., et al., *Systemic sclerosis: demographic, clinical, and serologic features and survival in 1,012 Italian patients.* Medicine (Baltimore), 2002. **81**(2): p. 139-53.
7. Artlett, C.M., *Animal models of scleroderma: fresh insights.* Curr Opin Rheumatol, 2010. **22**(6): p. 677-82.
8. Yamamoto, T., *Animal model of systemic sclerosis.* J Dermatol, 2010. **37**(1): p. 26-41.
9. Baxter, R.M., et al., *Analysis of the tight skin (Tsk1/+) mouse as a model for testing antifibrotic agents.* Lab Invest, 2005. **85**(10): p. 1199-209.
10. Green, M.C., H.O. Sweet, and L.E. Bunker, *Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton.* Am J Pathol, 1976. **82**(3): p. 493-512.
11. Szapiel, S.V., et al., *Hereditary emphysema in the tight-skin (Tsk/+) mouse.* Am Rev Respir Dis, 1981. **123**(6): p. 680-5.
12. Christner, P.J., et al., *The tight skin 2 mouse. An animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration.* Arthritis Rheum, 1995. **38**(12): p. 1791-8.
13. Peters, J. and S.T. Ball, *Tight skin-2 (Tsk2).* Mouse News Letter, 1986. **74**: p. 91-92.
14. Siracusa, L.D., et al., *A tandem duplication within the fibrillin 1 gene is associated with the mouse tight skin mutation.* Genome Res, 1996. **6**(4): p. 300-13.
15. Christner, P.J., et al., *A high-resolution linkage map of the tight skin 2 (Tsk2) locus: a mouse model for scleroderma (SSc) and other cutaneous fibrotic diseases.* Mamm Genome, 1996. **7**(8): p. 610-2.
16. Barisic-Dujmovic, T., I. Boban, and S.H. Clark, *Regulation of collagen gene expression in the Tsk2 mouse.* J Cell Physiol, 2008. **215**(2): p. 464-71.
17. Christner, P.J., et al., *Transcriptional activation of the alpha1(I) procollagen gene and up-regulation of alpha1(I) and alpha1(III) procollagen messenger RNA in dermal fibroblasts from tight skin 2 mice.* Arthritis Rheum, 1998. **41**(12): p. 2132-42.
18. Gentiletti, J., et al., *Demonstration of autoimmunity in the tight skin-2 mouse: a model for scleroderma.* J Immunol, 2005. **175**(4): p. 2418-26.
19. Lemaire, R., et al., *Fibulin-2 and fibulin-5 alterations in tsk mice associated with disorganized hypodermal elastic fibers and skin tethering.* J Invest Dermatol, 2004. **123**(6): p. 1063-9.
20. Sargent, J.L., et al., *A TGFbeta-responsive gene signature is associated with a subset of diffuse scleroderma with increased disease severity.* J Invest Dermatol, 2010. **130**(3): p. 694-705.
21. Walters, R., M. Pulitzer, and H. Kamino, *Elastic fiber pattern in scleroderma/morphea.* J Cutan Pathol, 2009. **36**(9): p. 952-7.
22. Quaglino, D., Jr., et al., *Connective tissue in skin biopsies from patients suffering systemic sclerosis.* J Submicrosc Cytol Pathol, 1996. **28**(2): p. 287-96.
23. Jimenez, S.A., A. Millan, and R.I. Bashey, *Scleroderma-like alterations in collagen metabolism occurring in the TSK (tight skin) mouse.* Arthritis Rheum, 1984. **27**(2): p. 180-5.
24. Vaalamo, M., et al., *Enhanced expression of human metalloelastase (MMP-12) in cutaneous granulomas and macrophage migration.* J Invest Dermatol, 1999. **112**(4): p. 499-505.

## TABLES

Table 1. Cyclical gene expression in skin of female and male Tsk2/+ mice over time

| | Female mice | | | | Male mice | | | |
|---|---|---|---|---|---|---|---|---|
| Transcript | 2 week | 4 week | 10 week | 17+ week | 2 week | 4 week | 10 week | 17+ week |
| COL1A1 | ~ | 5.7 | ~ | ~ | ~ | ~ | 1.8 | ~ |
| COL3A1 | ~ | 7.7 | ~ | ~ | ~ | 0.3 | 2.0 | ~ |
| COL5A2 | ~ | 4.3 | ~ | ~ | 1.7 | 0.3 | 2.2 | ~ |
| FBN1 | ~ | 2.7 | ~ | 2.8 | ~ | ~ | 3.4 | ~ |
| ELN | ~ | 12.6 | ~ | 2.3 | 2.7 | ~ | | ~ |
| FBLN2 | ~ | 2.6 | ~ | 2.1 | 1.8 | ~ | 3.2 | ~ |
| FBLN5 | ~ | 1.8 | ~ | ~ | ~ | ~ | ~ | ~ |
| ACTA2 | ~ | 4.9 | ~ | 2.2 | 3.1 | ~ | ~ | ~ |
| COMP | ~ | 3.5 | ~ | ~ | 1.7 | 1.4 | ~ | ~ |
| SPARC | 0.5 | 5.6 | ~ | ~ | ~ | ~ | 3.1 | ~ |

Legend: mRNA expression levels shown as a ratio of Tsk2/+ expression compared to WT mice. RNA was extracted from skin, cDNA was synthesized, and expression level was measured by qRT-PCR. Significant increases or decreases of Tsk2/+ vs WT skin was calculated by student's t-test. The table shows only significant increases or decreases ($p<0.05$) of expression levels and the magnitude of change is given. ~ represents no difference. Tsk2/+ females have significant increases in mRNA expression levels at 4 and 17+ weeks of age. Tsk2/+ males have significant increases in mRNA expression levels at 2 and 10 weeks of age. n=4-6 mice per group.

## FIGURES AND FIGURE LEGENDS

**Figure 1.** Body mass and footpad thickness comparison by age between Tsk2/+ and WT littermates (A-C). Mice were weighed and footpad thickness was measured weekly. **A,** Body mass was compared directly over time. **B** and **C,** Footpad thickness was binned by weight and compared by ages 4-6 weeks (**B**) and 10-12 weeks (**C**). Hydroxyproline content of the footpad by age between Tsk2/+ and WT littermates. **D,** Hydroxyproline (µg)/weight (µg) was determined from footpads. **A-C**, N>32 mice per group; **D,** N=9-12 mice per group.* p<0.05, **p<0.01, ***p<0.001.

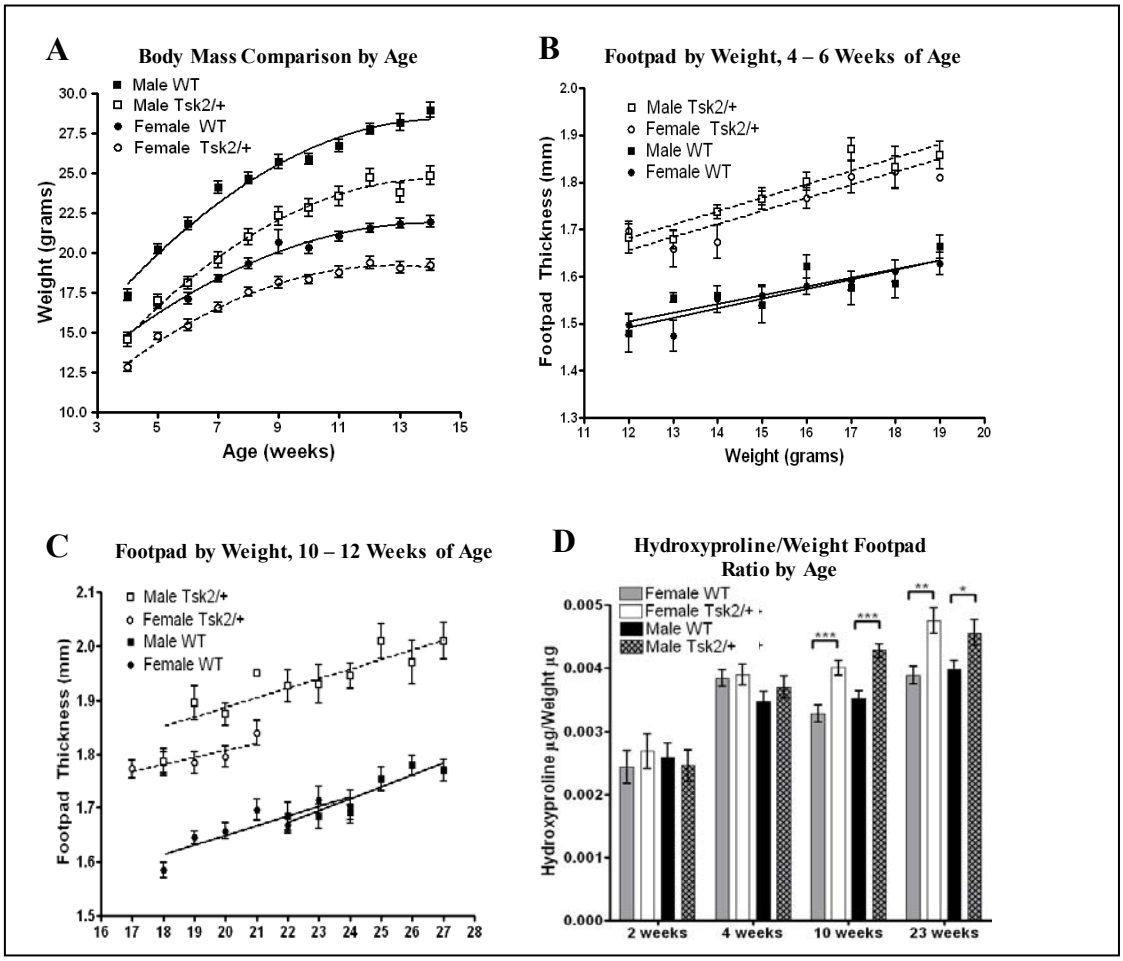

**Figure 2. <B&W>** Collagen content in skin. **A,** Skin samples were obtained from the lower dorsal back and processed for paraffin embedding. Samples were stained with Masson's Trichrome Blue stain and collagen percentage per HPF was calculated. Stained skin sections over time (10X magnification). **B**, Quantitative percentage of collagen per HPF over time. **C,** Hydroxyproline content of skin by age between Tsk2/+ and WT littermates. 8mm biopsy punches were used to remove a standard area of skin per mouse at 2, 4, 10, and 23 weeks of age and hydroxyproline content was determined. **B,** n=3-4 mice per group, 4-6 images per slide; **C,** n=9-12 mice per group. * p<0.05, **p<0.01, ***p<0.001.

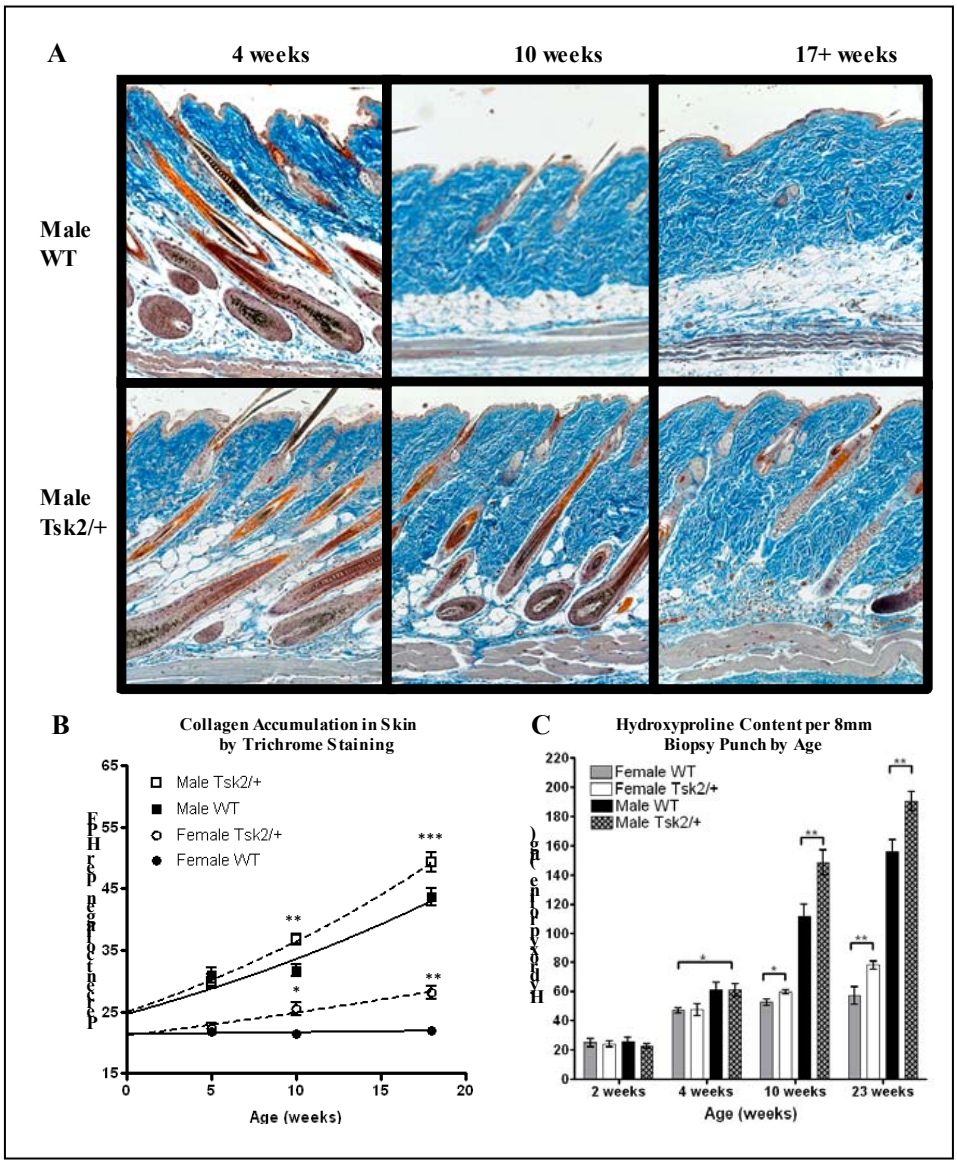

**Figure 3.** Infiltrating cell populations in Tsk2/+ and WT male mice. Skin samples were obtained from the lower dorsal back and processed for paraffin embedding and stained with toluidine blue for dermal mast cells (**A**) or anti-CD3 antibody for epidermal CD3+ T cells (**B**). The total number of cells per HPF were counted. Significance was calculated by student's t-test. n=4-6 mice per group, 5-9 HPFs per slide. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

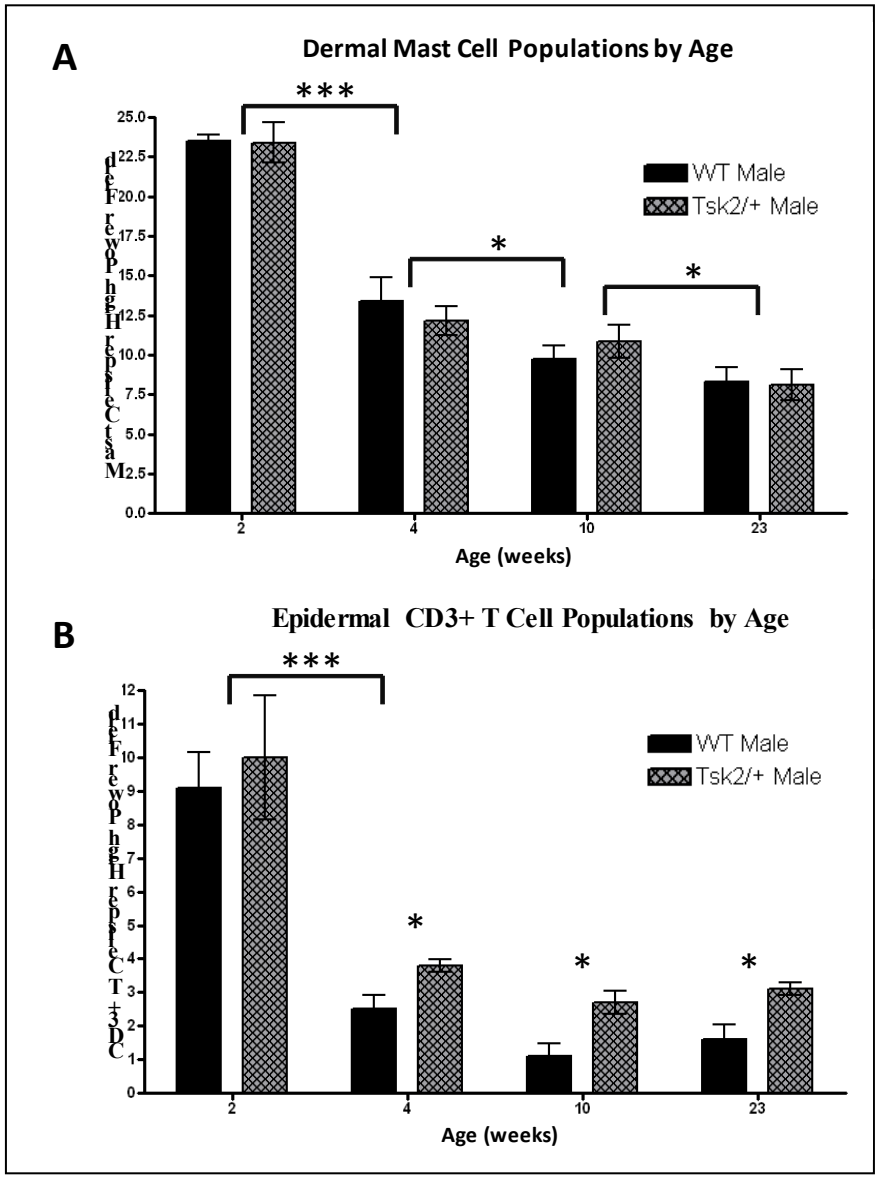

**Figure 4 <B&W>.** TGF-β1 and αSMA positive cells in Tsk2/+ and WT male mice. Skin samples were obtained from the lower dorsal back and processed for paraffin embedding and evaluated by IF for the presence of TGF-β1 (A) or αSMA positive cells (B). The total number of cells per HPF were counted. Significance was calculated by student's t-test. n=3-4 mice per group, 5-9 HPFs per slide. * p<0.05, **p<0.01, ***p<0.001.

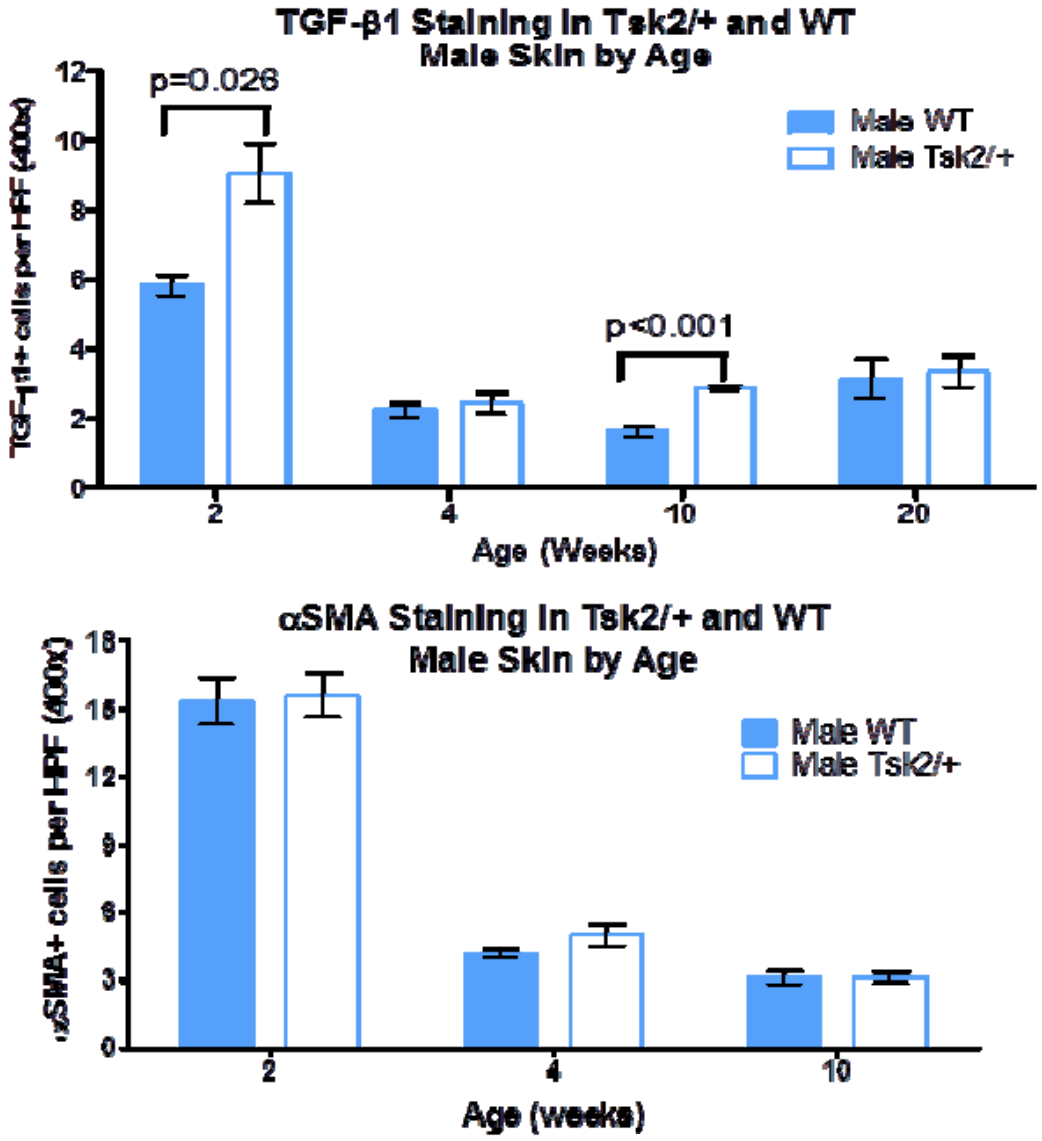

**Figure 5. <color>** Skin samples were obtained from the lower dorsal back and processed for paraffin embedding. Samples were stained with Weigert's Resorcin Fuchsin Stain and elastic fiber number per bright field was calculated. **A,** Elastic fiber WT (left), Tsk1/+ (middle) and Tsk2/+ (bottom) males at 5 weeks of age (40x magnification) shown between the hypodermal muscle (M) and the hypodermal connective tissue (HD). Distinct elastin fibers are marked with arrows. **B,** Elastic fibers in the dermis from WT (left) and Tsk2/+ (right) females at 5 weeks of age. **C,** Quantitative amount of elastic fibers per HPF over time. n=4-6 mice per group, 5-9 HPFs per slide. * p<0.05, **p<0.01, ***p<0.001.